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# Protective effects of *Ecklonia cava* extract on the toxicity and oxidative stress induced by hair dye in in-vitro and in-vivo models

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**Abstract** Oxidative hair dyes containing ρ-phenylenediamine (PPD) are reported to induce an allergic reaction by promoting oxidative stress when absorbed through the skin. Despite the associated risk, these hair dyes remain popular owing to their convenience and sharpness of color. This makes it important to minimize the cytotoxicity and oxidative stress induced by PPD-containing hair dyes. *Ecklonia cava* extract has been evaluated in different studies for its protective effects against external stress in fibroblasts and keratinocytes. Our study was aimed at using in-vitro and in-vivo models to investigate the extract's effects on cytotoxicity of and oxidative stress induced by PPD-containing hair dyes. Analysis of CIEL*a*b* Color space was first used to determine the range of *E. cava* extract that would not interfere with the coloring ability of the dye upon addition. Subsequently, the set ranges of *E. cava* extract (5% and 7%) were added to the hair dye and their toxicity assessed by evaluating the viability of fibroblasts and keratinocytes. The effects on developmental phenotypes and induction of oxidative stress by hair dye were evaluated and compared with those of hair dyes containing different contents of *E. cava* extract using an in-vivo zebrafish model. Our results showed that *E. cava* extract in hair dye could significantly decrease the cytotoxicity and levels of oxidative stress caused by hair dyes containing PPD in both in-vitro and in-vivo models. These results suggest that the addition of 7% *E. cava* extract to 250 μg/mL hair dye does not interfere with the coloring ability of the dye while showing significant protective effects against the hair dye. The study proposes that the use of *E. cava* extract as an adduct to hair dyes containing PPD reduces the cytotoxicity and oxidative stress induced by these hair dyes.

**Keyword**: hair dye; *Ecklonia cava*; cytotoxicity; oxidative stress; in-vitro and in-vivo models

## 1 INTRODUCTION

Hair dyes and many chemicals used in the hair dyeing process can cause allergic contact dermatitis upon contact with the skin and their topical application may result in toxic effects (IARC, 1993, Nohynek et al., 2010; Handa et al., 2012). In permanent oxidative hair dyes, aromatic amines (e.g. ρ-phenylenediamines (PPDs) and ρ-aminophenols) are oxidized by hydrogen peroxide as primary intermediates before reacting with couplers to produce dyes (Monnais, 1995; Corbett, 1998). Hair dye ingredients that contain highly reactive molecules have allergenic potency. Hair dyes containing PPD are potent, toxic, and cause allergic sensitization that can induce severe contact hypersensitivity in living organisms (SCCNFP, 2002; Rubin et al., 2010). Although the potential risk has been known, PDD-containing hair dyes are still the most widely used dyes for permanent hair coloring worldwide (Stanley et al., 2005). Therefore, with the proven toxicity of the ingredients and hazard associated with using these hair dyes,

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controlling and minimizing the toxicity of hair dyes containing PPD at the manufacturing level are an important consideration for hair dye producers. For this reason, the addition of agents to hair dyes that can reduce the toxicity of PPD is a practical approach to achieving safer hair dye formulations.

*Ecklonia cava* is a marine alga known to have various bioactive compounds and derivatives including phlorotannins and polyphenols that exert a protective effect against cellular toxicity and oxidative stress in in-vitro and in-vivo experiments (Heo et al., 2009; Ko et al., 2011; Bak et al., 2013; Kim et al., 2014). The protective effects of exogenously-derived antioxidants belonging to the polyphenol class from *E. cava* on UV- or free radical-induced fibroblast and keratinocyte damage (Joe et al., 2006; Ko et al., 2011) have led us to investigate the ability of *E. cava* to reduce the toxicity of hair dyes and the PPD ingredient.

Through in-vitro and in-vivo experiments, the study assessed the cytotoxicity and oxidative stress caused by hair dyes and evaluated the protective effect of *E. cava* extract at concentrations (5% and 7%) that would not interfere with the dye's coloring ability. Our results suggest that *E. cava* extract can be used as an adduct to hair dyes containing PPD to reduce cytotoxicity and oxidative stress.

## 2 MATERIAL AND METHOD

### 2.1 Chemicals

*Ecklonia cava* containing (43±1.3)% of polyphenol was extracted in 70% ethanol as previously described elsewhere (Ko et al., 2011). 2,7-Dichlorofluorescein diacetate (DCF-DA), diaminofluorophore4-amino-5-methylamino-2′7′-difluorofluorescein diacetate (DAF-FM DA), acridine orange (AO), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2 Preparation of hair dye with *E. cava* extracts

The hair dye containing 3.5% of ρ-phenylenediamine (PPD, CAS No. 106-50-3) used in the formulation of black color dye was prepared using the proportions recommended by the manufacturer for commercial use (A. G. Tech, Aqua Green Technology Co. Ltd., Republic of Korea). To prepare the hair dye containing *E. cava*, the dye with desired concentrations (250, 500, and 1 000 μg/mL) was mixed with *E. cava* (dissolved in DMEM) to yield final percentages of 5%, 7%, 9%, and 11%.

### 2.3 Dyeing hair

#### 2.3.1 Hair dye

Bleached blonde hair swatches were obtained from Fischbach & Miller (Laupheim, Germany). The swatches were flat, weighing ~1 g, and were 10 cm long. The swatches were treated with hair dye containing different concentrations of *E. cava* extract (5% and 7%) and mixed with the oxidizing agent, 6% $H_2O_2$ (L'Oreal Korea), at the ratio 1:60 for 10 min followed by rinsing. All hair swatches were washed with a commercial shampoo prior to dyeing and drying.

#### 2.3.2 Color analysis

The CIEL*a*b* color space (L*, a*, b* (Jones et al., 2016)) having dimensions closely resembling the color channels of the human visual system was used to represent the colors of each dyed hair in Adobe Photoshop CS3. The three orthogonal dimensions of this color space were light-dark (L*), red-green (a*), and yellow-blue (b*), and, the pixel values for CIEL*a*b* within dyed hair was randomly chosen ten-times and averaged for each hair dye containing different concentrations of the *E. cava* extract. The pixel values ranged from 0 (L*, black; a*, green; b*, blue) to 255 (L*, white; a*, red; b*, yellow).

### 2.4 In vitro assay of cell viability

Human dermal fibroblasts (HDF cells) and keratinocytes (HaCaT cells) were maintained in DMEM supplemented with 10% heat-inactivated FBS, streptomycin (100 μg/mL), and penicillin (100 unit/mL) at 37°C with 5% $CO_2$. Cells were sub-cultured at 3-day intervals (80% confluence) using trypsin-EDTA. For the viability assay, HDF and HaCaT cells were seeded in 96-well plates at a density of $1\times10^4$ cells per well and were treated with hair dye and hair dye containing different concentrations of *E. cava* extract for 24 h. Hair dye at concentrations of 250, 500, and 1 000 μg/mL with 0, 5%, and 7% *E. cava* extract were tested. Fifty microliters of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent was added to each well and incubated for another 2 h. Cell viability was determined by measuring the optical density (OD) of the solution at 540 nm using a microplate reader (Tecan Austria GmbH, Salzburg, Austria).

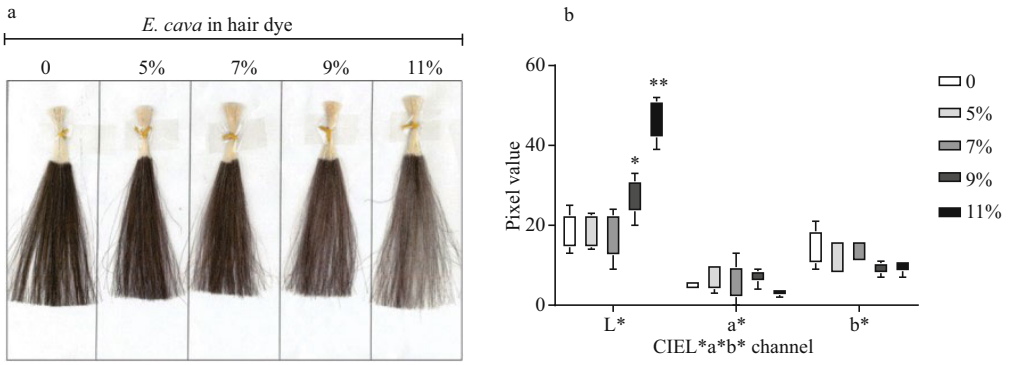


**Fig.1 Pixel values for CIEL*a*b* of hair dyed with the hair dye containing different concentrations (0, 5%, 7%, 9%, and 11%) of *E. cava* extract**

$^{*}P<0.05$, $^{**}P<0.01$ compared with the value (L*a*b*) of the hair dye without *E. cava* extract.

### 2.5 In-vivo experiment

#### 2.5.1 Maintenance of parental zebrafish and collection of embryos

Adult zebrafish were purchased from a commercial dealer (Seoul aquarium, Korea). They were kept separately in 3 L acrylic tanks at 28.5°C, with a 14 h/10 h light/dark photocycle, fed 3 times per day, 6 days per week, with Tetramin flake food supplemented with live brine shrimps. Embryos were obtained by natural spawning induced when the light was switched on in the mornings. Collections of embryos were completed within 30 min with the embryos that achieved normal development and reaching the blastula phase kept in the embryo medium for further analysis. The experiment with zebrafish was approved by the Animal Care and Use Committee of the Jeju National University (No. 2017-0001).

#### 2.5.2 Treatment design and evaluation of developmental phenotypes

The embryos ($n$=15) were transferred to individual wells of 12-well plates containing 900 μL embryo media and exposed to hair dyes (250 and 500 μg/mL) containing different concentrations of *E. cava* (5% and 7%). Embryo mortality was monitored daily for up to 7 days post-fertilization (dpf).

At 3 dpf, heartbeat rate of both the atrium and ventricle in zebrafish was recorded for 1 min and the yolk sac edema was measured using ISCapature to calculate the pixels of the pericardium area under a light microscope (Olympus, Tokyo, Japan).

Survival rate was calculated based on the rate of surviving larvae divided by the total number of embryos in each replicate. Morphological changes were observed every 24 h for 2 dpf under a microscope.

#### 2.5.3 Analysis of oxidative stress indexes

Generation of NO in the zebrafish larvae was evaluated using a fluorescent probe dye, DAF-FMDA, and levels of ROS were detected using an oxidation-sensitive fluorescent probe dye, DCF-DA. Embryos were exposed to hair dyes (250 and 500 μg/mL) containing different concentrations of *E. cava* for 3 dpf, transferred to a 24-well plate and treated with embryo medium containing 10 μmol/L DAF-FMDA or 20 μg/mL DCF-DA in the dark at 28°C for 1 and 2 h respectively. After incubation, the larvae were washed with embryo media twice and anesthetized with 0.03% MS-222 for 3 min before visualization. The images of stained embryos were observed using a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan).

### 2.6 Statistical analysis

The experiments conducted in this study were statistically analyzed using one-way analysis of variance (one-way ANOVA) and Dunnett's multiple comparison tests (in SPSS 12.0 statistical software). Significant differences between treatment means were determined by Duncan's multiple range tests where $^{*}P<0.05$ and $^{**}P<0.01$ were considered statistically significant.

## 3 RESULT

### 3.1 Determination of appropriate content of *E. cava* extract in hair dye

To check the ability of hair dye containing different concentrations of *E. cava* extract (5%, 7%, 9%, and 11%) in dyeing hair, we dyed the swatches with the hair dyes. Figure 1a shows the gradual lightening of swatches after dyeing with the hair dyes containing

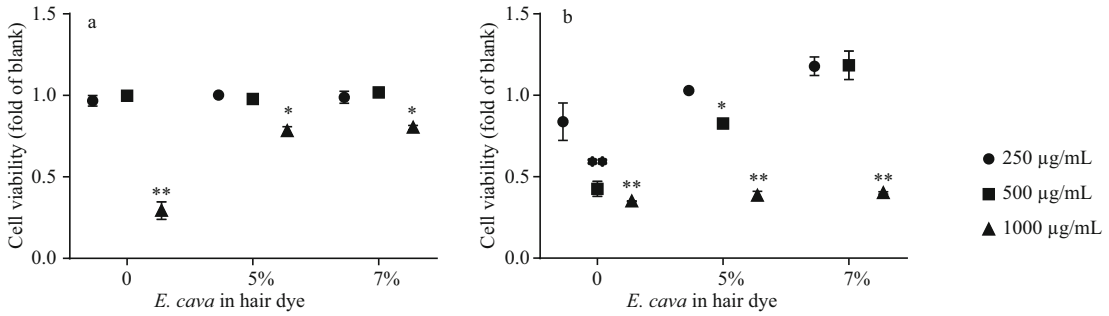


**Fig.2 Dose-dependent effect (250, 500, and 1 000 μg/mL) of hair dye with 0, 5%, and 7% *E. cava* extract on the viability of HDF (a) and HaCaT (b) cells**

Each cell line was incubated with the hair dye with different concentrations of *E. cava* extract for 24 h. Cell viability was determined by the MTT assay. The data are shown as mean±SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with that of the blank (no hair dye).

increasing concentrations of *E. cava* extract. These changes were evaluated using the CIEL*a*b* color space (Fig.1b). L*a*b* color space corresponds to the perceived color differences between stimuli (Brainard, 2003). The L* value of the swatches dyed with the hair dye containing 5% and 7% *E. cava* extract did not show any significant difference from that of the swatches dyed with the hair dye without *E. cava* extract (0, 18.6 of $P_L$). However, this value increased significantly with hair dyes containing 9% and 11% *E. cava* extract (27.2 and 46.6 of $P_L$, respectively), indicating a perceptual lighter color than the color achieved with the hair dye without *E. cava* extract (0) (Fig.1b). Therefore, we set the range of *E. cava* extract concentration to 5% and 7%, which did not interfere with the dying ability of the hair dye.

### 3.2 Assessment of the protective effect of *E. cava* extract in hair dye on the cytotoxicity induced by hair dye in human skin cells

To evaluate the protective effect of *E. cava* extract on the cytotoxicity induced by hair dye containing 3.5% PPD, we set the highest dose of hair dye to 1 000 μg/mL, which contains 35 μg/mL PPD, a concentration similar to the half maximal effective cytotoxic concentration ($EC_{50}$) for HaCaT cells (Zanoni et al., 2015). The cells were treated with different concentrations (250, 500, and 1 000 μg/mL) of hair dye containing *E. cava* extract (5% and 7%) for 24 h and cell viability was subsequently determined by the MTT assay (Fig.2). In HDF cells, the treatment with 1 000 μg/mL hair dye significantly decreased cell viability by 0.29 fold compared with that of the blank, which is the non-hair dye treatment group ($^{**}P<0.01$). This decrease in cell viability was recovered to 0.78 and 0.80 folds compared with that of the blank in the cells treated with hair dye containing 5% and 7% *E. cava* extract, respectively ($^{*}P<0.05$). With 250 and 500 μg/mL hair dye treatments, no significant decrease in cell viability was observed. Furthermore, there was no significant difference between the groups treated with hair dye and hair dye containing *E. cava* extract. In HaCaT cells, the cytotoxicity induced by 500 μg/mL hair dye was reduced with the hair dye containing 5% *E. cava* extract to 0.82 fold compared with that of the blank. With the hair dye containing 7% *E. cava* extract, there was no cytotoxicity (cell viability was fully recovered). These results suggest the protective effect of 5% and 7% *E. cava* extract on the cytotoxicity induced by the increase in the concentration of hair dye to up to 500 μg/mL.

### 3.3 Assessment of the effect of *E. cava* extract in hair dye on the developmental phenotypes of zebrafish

To evaluate the differential effect of hair dye and hair dye containing *E. cava* extract on the embryonic development of zebrafish, the zebrafish were exposed to hair dyes (250 and 500 μg/mL) containing *E. cava* extract (5% and 7%) and were observed at different time points for 7 dpf. As shown in Fig.3a, 250 μg/mL of hair dye containing *E. cava* extract (5% and 7%) did not significantly cause embryo death for 7 d, whereas, the hair dye without *E. cava* extract caused a 30% decrease in survival at 1 dpf and approximately 50% decrease at 7 dpf. Changes in the developmental morphology were observed with 250 μg/mL of hair dye with different concentrations of *E. cava* extract for 2 dpf (Fig.3b). The hair dye with no *E. cava* extract caused embryo death at 1 dpf (top panel, Fig.3b) and delayed embryo hatching at 2 dpf (bottom panel, Fig.3b); however, no effect on embryo hatching was observed with the hair dye containing 5% and 7%

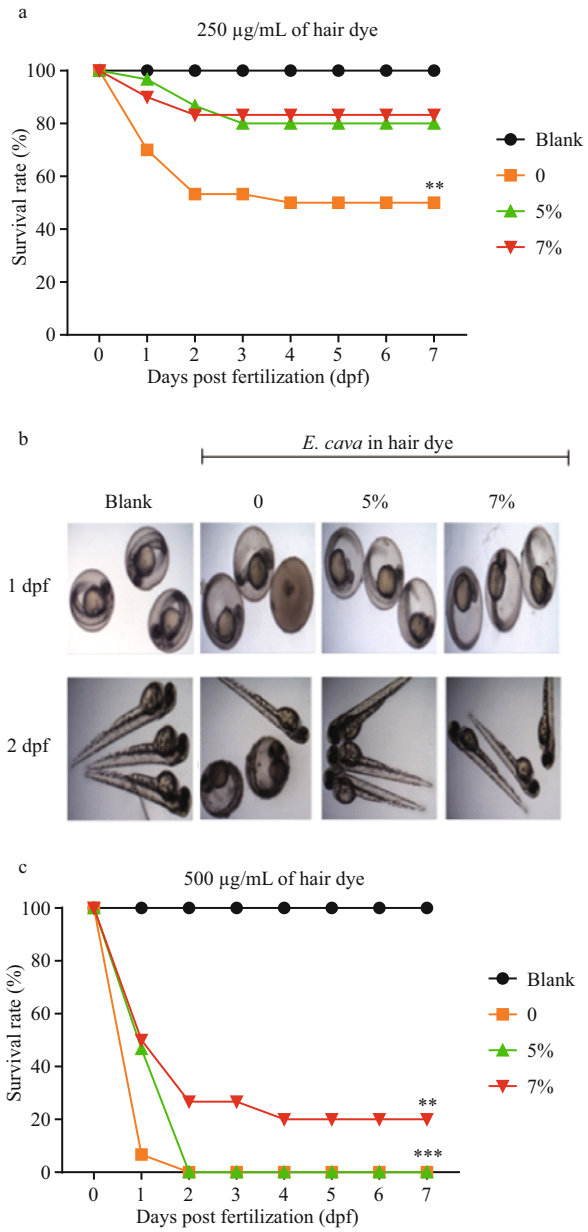


**Fig.3 Dose-dependent effect (250 and 500 μg/mL) of hair dye containing 0, 5%, and 7% *E. cava* extract on zebrafish survival rate during 7 dpf**

Changes in survival rate and developmental morphology with 250 (a and b) and 500 μg/mL (c) hair dye with *E. cava* extract were observed. The morphological phenotype was captured at 1 and 2 dpf. The values significantly different from those of the blank at 7 dpf are indicated with asterisks (*$P$<0.05, **$P$<0.01, ***$P$<0.005).

*E. cava* extract. The embryo mortality rate with 500 μg/mL of hair dye containing 0 and 5% *E. cava* extract was 100%, which was reduced to 80% with 7% *E. cava* extract (**$P$<0.01) compared with that of the blank control (Fig.3c).

The heart rate and yolk sac edema were measured in zebrafish embryos treated with 250 μg/mL hair dye containing *E. cava* extract (5% and 7%) (Fig.4). The

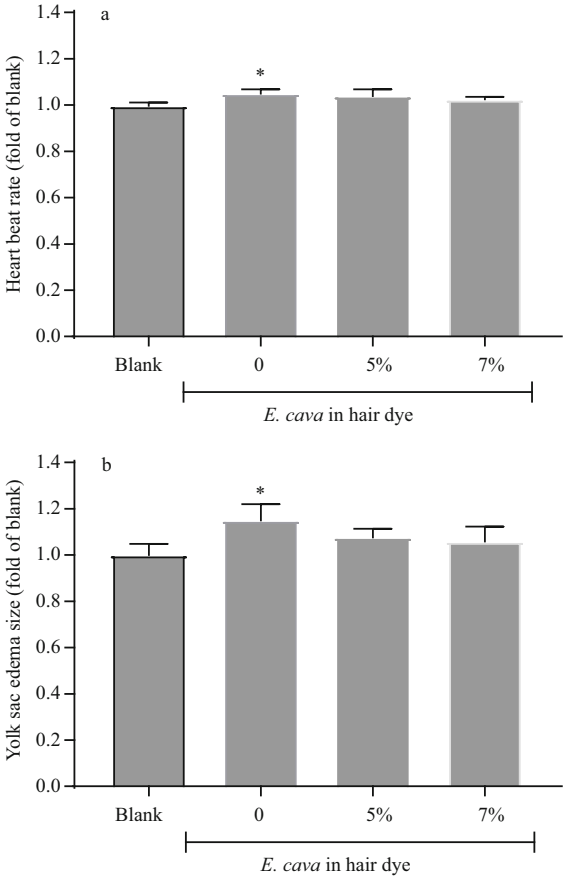


**Fig.4 Effects of 250 μg/mL hair dye with 0, 5%, and 7% *E. cava* extract on the heart rate (a) and yolk sac edema size (b) at 3 dpf in zebrafish**

The values significantly different from those of the blank are indicated with asterisks (*$P$<0.05).

heart rate of control zebrafish (blank) was 91±0.60 beats per min, and it was significantly increased to 96±0.95 by the treatment with hair dye ($P$<0.05). However, all hair dyes containing *E. cava* extract did not show any significant difference in heart rate compared with that of the blank control (Fig.4a). The abnormality in the size of yolk sac edema induced by hair dye was also reduced in all the groups treated with hair dye containing *E. cava* extract (Fig.4b).

### 3.4 Assessment of the effect of *E. cava* extract in hair dye on oxidative stress in zebrafish

Reactive oxygen species (ROS) and nitric oxide (NO) are the major indexes of oxidative stress caused by the imbalance in the redox state of a cell or tissue. They are well-described biomarkers to determine the oxidative stress profile of an organism (Kang et al., 2015; Li et al., 2017). Exposure of zebrafish larvae to 250 μg/mL hair dye without *E. cava* extract for 3 dpf resulted in a significant increase in DCF fluorescence

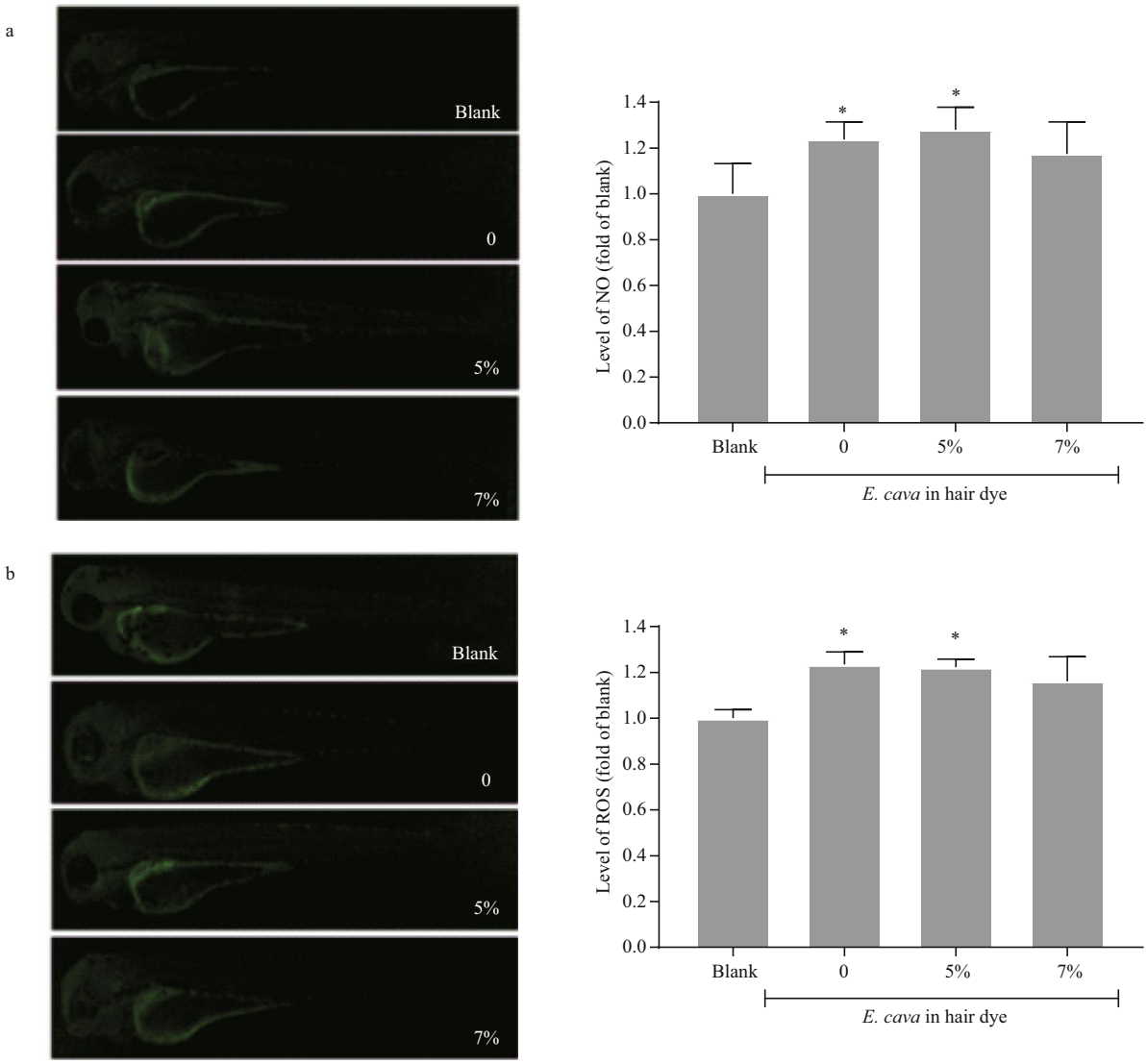


**Fig.5 Oxidative stress in zebrafish embryos exposed to 250 μg/mL hair dye with 0, 5%, and 7% of *E. cava* extract at 3 dpf, and the level of ROS (a) and NO (b)**

The values significantly different from those of the blank are indicated with asterisks (*$P$<0.05).

compared with that of the blank (no hair dye treatment), indicating ROS generation due to the oxidative stress induced by hair dye (Fig.5a). The increased level of ROS was significantly decreased following the addition of 7% *E. cava* extract to the hair dye and showed no significant difference from that of the blank (Fig.5a). The effect of *E. cava* extract in 250 μg/mL hair dye on NO level was also investigated using DAF-FM fluorescence (Fig.5b). The treatment of hair dye significantly induced the level of NO determined by increased DAF-FM fluorescence, which was reduced by the addition of 7% *E. cava* in the hair dye (Fig.5b).

## 4 DISCUSSION

Hair dyes are used globally for cosmetic purposes, with a market that is rapidly growing and is expected to reach USD 29.14 billion by 2019 (Technavio Research, 2016). Besides the conventional use by older people, dyeing of hair has become popular among both men and women as a fashion trend. Among hair dyes, oxidative hair dyes are the most commonly used dyes due to their stability and have a market share of approximately 80% (Corbett, 1999). The oxidative hair dyes consist of primary intermediates (e.g., ρ-phenylenediamines (PPDs) and ρ-aminophenols) and couplers (e.g., m-aminophenols and m-hydroxyphenols), which dye the hair in the presence of peroxide (Monnais, 1995). ρ-Phenylenediamine, which is used to obtain intense black shade, is an aromatic amine that is oxidized by hydrogen peroxide ($H_2O_2$), and it subsequently reacts

with a coupler resulting in the desired color within the hair shaft (Corbett, 1999). Recently some studies have suggested the induction of oxidative stress and DNA damage by PPD used in hair dye via the formation of highly reactive hydroxyl radicals upon skin exposure in human keratinocytes (Aeby et al., 2009; Corsini et al., 2013; Gibbs et al., 2013; Zanoni et al., 2015). Zanoni et al. (2015) also reported the cytotoxic effects of PDD (with a half-maximal cytotoxic concentration of 39.37 μg/mL) and the induction of oxidative stress after the addition of $H_2O_2$ to PDD in keratinocytes. Their study and others have emphasized on the substantial risk of cytotoxicity and oxidative stress induced by PPD in hair dye on human skin cells, which are directly exposed to hair dyes. To address this issue, we evaluated the effect of *E. cava* extract in the hair dye on the toxicity and oxidative stress induced by the hair dye in in vitro and in vivo models.

*Ecklonia cava*, a marine alga, has been reported to possess various phlorotannins that act as free radical scavengers and thus have attracted attention owing to their protective effects on human keratinocyte and dermal fibroblasts by reducing the oxidative stress induced by UV-B (Joe et al., 2006; Heo et al., 2009; Pallela et al., 2010; Ko et al., 2011). Furthermore, Bak et al. (2013) showed that *E. cava* extract promotes hair growth via the stimulation of human dermal papilla cells and outer root sheath cells.

In this study, we added different concentrations (5%, 7%, 9%, and 11%) of *E. cava* extract to hair dye containing 3.5% PPD and evaluated their ability to dye hair using the CIEL*a*b* color space. L*a*b* color space has been designed such that the differences between stimuli project the perceived color difference between the stimuli (Brainard, 2003). Between the hair dye and hair dye containing 5% and 7% *E. cava* extract, no significant difference in pixel values for red-green (a*) and yellow-blue (b*) was observed; however, the pixel value of light-dark (L*, 18.6 pixel value with normal hair dye) increased to 27.2 and 46.6 pixels with the addition of 9% and 11% *E. cava* extract, respectively. That is, the color became lighter than that of the normal hair dye without *E. cava* extract. With 5% and 7% *E. cava* extract in hair dye, no significant color difference in any of the three channels was observed compared with that of the hair dye without *E. cava* extract, suggesting a similar potency as hair dye. Therefore, we examined the changes in the cytotoxicity of hair dye with the addition of 5% and 7% *E. cava* extract.

The crosstalk between fibroblasts and keratinocytes involves the activity of a number of growth factors and cytokines. Stress on epidermal skin, including keratinocytes, leads to the generation of several metabolites that scavenge free radicals and stimulate anti-oxidant enzymes, which additionally influence fibroblast cells (Zanoni et al., 2015; Varma et al., 2016). Therefore, further studies on the effect of hair dye and its additive on the reciprocal interaction between fibroblasts and keratinocytes are warranted.

To investigate the effect of *E. cava* in the hair dye on the cytotoxicity induced by hair dye, we examined the viability of human fibroblasts and keratinocytes. We used 1 000 μg/mL hair dye containing 3.5% PPD (equal to 35 μg/mL of PPD) as the highest concentration, which is the cytotoxic $EC_{50}$ of PPD reported by Zanoni et al. (2015). Although the viability of fibroblasts was not affected by the treatment with 250 and 500 μg/mL hair dye, cytotoxicity was significantly induced in keratinocytes by 500 μg/mL hair dye, which were protected with the increase in the content of *E. cava* in the hair dye.

The protective effect of *E. cava* extract on the developmental changes induced by hair dye in in vivo zebrafish model was observed (Figs.3, 4). The survival rate and hatching rate of fish are indexes used to evaluate developmental toxicology, as a combination of biochemical and physical mechanisms. These indexes can be affected by abnormal heartbeat rate and yolk sac edema size (Asharani et al., 2008). The heart is one of the first functional organs developed in zebrafish. The heartbeat rate is an important toxicological end-point due to the ability of zebrafish to actively regulate their cardiac output in response to changes in the environment. It is a behavioral response that is considerably similar to that observed in mammals (Mann et al., 2010). In the present study, 250 μg/mL hair dye caused a significant reduction in embryo survival and hatching rate at 2 dpf (*$P$<0.05); however, 5% and 7% *E. cava* extract in hair dye recovered this reduction, and only showed a marginal decrease (not statistically significant) in the survival rate and hatching rate compared with those of the control group. Similarly, the induced heart rate and increased yolk sac edema size caused by hair dye were recovered with the addition of *E. cava* extract, and there was no significant difference from those of the non-treated zebrafish (blank). Our findings indicate that the addition of *E. cava* extract to hair dye can reduce the cytotoxicity induced by hair dye in in vitro and in vivo models.

The effect of *E. cava* extract in hair dye on the oxidative stress induced by hair dye was also evaluated in the zebrafish model. The level of ROS and NO in zebrafish was assessed after 3 d of exposure to hair dye and compared with those of the treatment with hair dyes containing different concentrations of *E. cava* extract. The results showed that the increase in the level of ROS and NO induced by 250 μg/mL hair dye was reduced by the addition of *E. cava* extract.

## 5 CONCLUSION

In summary, our results revealed 1) the dyeing ability of hair dye containing different concentrations of *E. cava* extract; 2) the protective effects of *E. cava* in hair dye on the cytotoxicity induced by hair dye; and 3) the oxidative stress induced by 250 μg/mL hair dye in human fibroblasts and keratinocytes and zebrafish models. This study shows the potential of *E. cava* extract as an adduct to hair dye in order to reduce the cytotoxicity and oxidative stress induced by hair dye.

## 6 DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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